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Changes in cytosolic pH and calcium of guard cells precede stomatal movements

(abscisic acid/kinetin/indoleacetic acid/*Paphiopedilum*/confocal scanning optical microscopy)

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ABSTRACT **Stomatal opening is induced by indoleacetic acid (IAA), cytokinins, and fusicoccin (FC), whereas stomatal closure is induced by abscisic acid (ABA). To test the effect of these growth regulators on guard cell cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) and pH (pH_{cyt}), epidermal strips were taken from the lower side of leaves of the orchid *Paphiopedilum tonsum* and were loaded with acetomethoxy-esterified forms of the Ca^{2+} indicator fluo-3 or the pH indicator 2′,7′-bis(2-carboxyethyl)-5(6)carboxyfluorescein. Basal $[Ca^{2+}]_{cyt}$ ranged from 0.05 to 0.3 μM and was 0.22 ± 0.015 (n = 21). Increases in both $[Ca^{2+}]_{cyt}$ and pH_{cyt} were observed in guard cells after application of 10–100 μM ABA to open stomata, and these preceded stomatal closure. The increase in $[Ca^{2+}]_{cyt}$ ranged from 1.5- to 3-fold and was seen in 7 of 10 experiments. Guard cell alkalinization began within 2 min of ABA treatment and continued for the next 8 min. The increase ranged from 0.04 to 0.3 pH unit and was seen in 13 of 14 experiments. Guard cell $[Ca^{2+}]_{cyt}$ increased, whereas pH_{cyt} decreased after treatment of closed stomata with IAA, kinetin, or FC. In response to 50–100 μM IAA, $[Ca^{2+}]_{cyt}$ increased 1.5- to 2-fold in all cases, and pH_{cyt} decreased 0.2–0.4 unit within 5 min in 7 experiments. Within 12 min, 10–100 μM kinetin caused $[Ca^{2+}]_{cyt}$ to increase in 28 of 34 experiments (1.3- to 2.5-fold) and pH_{cyt} fell 0.1–0.4 unit in 15 of 17 treatments. The response to 10–50 μM FC was similar in both time and magnitude. These results show that stomatal opening is accompanied by an increase in $[Ca^{2+}]_{cyt}$ and cytosolic acidification in the guard cells, whereas stomatal closure is preceded by an increase in $[Ca^{2+}]_{cyt}$ and cytosolic alkalinization in the guard cells. The order of these events is still uncertain, but changes in pH_{cyt} are correlated with stomatal movement, and these changes may be an important factor in the regulation of guard cell movement.**

The opening and closure of stomatal pores, which regulate gas exchange in leaves, are associated with large changes in K^+ levels in the guard cells (1). The K^+ content of the guard cells is increased during stomatal opening (1), which is induced by light (2), low CO_2 levels (3), humidity (4), indoleacetic acid (IAA) (5), fusicoccin (FC) (6), and cytokinins (7). Stomatal closure is associated with K^+ release from the vacuoles and efflux across the plasma membrane (1), and closure can be induced by elevated levels of CO_2 (2, 3), darkness (2), decreased humidity (2, 4), and abscisic acid (ABA) (8). It has been suggested that the K^+ efflux is preceded by a Ca^{2+} influx across the plasma membrane and thus by a rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) (1). Changes in $[Ca^{2+}]_{cyt}$ have been postulated to act as the second messenger in both stomatal opening and closure in response to plant growth regulators (for a review, see ref. 3). The evidence for a role of $[Ca^{2+}]_{cyt}$ in stomatal closure is greater than in stomatal opening, as increases of $[Ca^{2+}]_{cyt}$ in guard cells have been shown to precede stomatal closure (9–11). However, variable responses of guard cell $[Ca^{2+}]_{cyt}$ after ABA treatment have been reported (9–12). A pattern of spikes of $[Ca^{2+}]_{cyt}$ is seen in guard cell protoplasts as nonselective Ca^{2+}-permeable channels are activated in response to ABA (12). Guard cell $[Ca^{2+}]_{cyt}$ increases in response to ABA (9–11), but a range of changes (including no detectable increase) in $[Ca^{2+}]_{cyt}$ have been seen (11). As stomatal closure was observed consistently, it was suggested that an ABA-induced Ca^{2+}-independent mechanism also operated to close stomata (11). However, in *Vicia faba* guard cell protoplasts, an increase in $[Ca^{2+}]_{cyt}$ was detected in all cells that responded to ABA (12). This could reflect the heterogeneity of stomatal behavior in the leaf (for a review, see ref. 3).

IAA has also been shown to increase $[Ca^{2+}]_{cyt}$ in several cell types (but not as yet in guard cells), in maize (13, 14), and parsley (14). IAA decreases cytosolic pH (pH_{cyt}) (13–16), whereas ABA increases pH_{cyt} in maize and parsley cells (14). As both IAA and ABA increase $[Ca^{2+}]_{cyt}$ but have opposite effects on pH_{cyt} (14), we have suggested that this difference underlies the antagonistic effects of the two plant growth regulators. To test if these responses also occur in guard cells, we have used confocal scanning optical microscopy to monitor changes in $[Ca^{2+}]_{cyt}$ and pH_{cyt} in epidermal strips of the orchid *Paphiopedilum tonsum*. Guard cells of *Paphiopedilum* spp. lack chlorophyll (17) and thus are suitable for monitoring changes in fluorescence of the indicator dyes for Ca^{2+}, fluo-3, and for pH, 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Although changes in stomatal aperture of *Paphiopedilum* spp. are small, they have been shown to open in response to blue light (18) and to close in response to ABA (19), suggesting that the control mechanisms are the same as in other guard cells. In this paper, we report that acidification of guard cell cytosol preceded stomatal opening induced by kinetin, IAA, or FC, whereas alkalinization of guard cell cytosol occurred prior to stomatal closure in response to ABA. On the other hand, an increase in $[Ca^{2+}]_{cyt}$ was observed in response to ABA, kinetin, IAA, and FC. These results strongly suggest that pH_{cyt} is a further factor in the regulation of guard cell movement, and we discuss how $[Ca^{2+}]_{cyt}$ and pH_{cyt} might interact in this process.

MATERIALS AND METHODS

Plant Material. *Paphiopedilum tonsum* var. *curtisfolium* were grown in a potting mix [1-cm-diameter blue metal/0.5-cm-diameter pine bark/0.5-cm diameter quartz, 1:1:1 (vol/vol)] containing nutricote (13:5:9 N/P/K ratio). The plants were kept moist in subdued light in the glasshouse in a temperature regime ranging from 10°C to 28°C. The experiments reported were undertaken in spring to late summer. Epidermal strips were peeled from the abaxial (lower) surface

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Abbreviations: ABA, abscisic acid; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+}; FC, fusicoccin; IAA, indoleacetic acid; pH_{cyt}, cytosolic pH.

of expanded leaves of 2- to 3-year-old plants. More than 70% of the stomata of untreated strips were closed. Stomata with a pore width of >1.5–2 μm were considered as open, and >30 stomata from at least six epidermal strips were assessed 45–60 min after treatment. Stomatal movement is expressed as the difference between the mean percentage of open stomata and that of untreated controls. To promote stomatal opening prior to experiments with ABA or procaine, epidermal strips were submerged for 2.5–3 hr at 20–25°C in an "opening buffer" (pH 6.2) containing 10 mM Pipes (Sigma), 50 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, and 100 μM $CaCl_2$ under blue light [0.35 $E \cdot m^{-2} \cdot s^{-1}$, where E = Einstein (1 mol of photons)] and were aerated with house air passed through a column of self-indicating soda lime (Carbosorb; BDH). These conditions resulted in epidermal strips with 65–80% open stomata.

Dye Loading. The epidermal strips were loaded with the fluorescent probes by incubation with the acetoxymethyl esterified forms of fluo-3 or BCECF (Molecular Probes). The epidermal strips were incubated for 30 min in the dark at 20–24°C in incubation buffer (pH 6.2) containing 10 mM Pipes, 50 mM KCl, 1 mM $MgCl_2$, and 20 μM BCECF–acetoxymethyl ester. Incubations with 20–40 μM fluo-3/acetoxymethyl ester were usually for 1–2 hr, and uptake was further facilitated when 0.05% pluronic F-127 was present (14). Loading was terminated by rinsing the strips several times in incubation buffer lacking the dye. The period of incubation to load the dye resulted in some closure, but the majority of the stomata remained open in the strips previously exposed to blue light.

Confocal Scanning Optical Microscopy. The epidermal strips were viewed with a Nikon Optiphot microscope. Intracellular fluorescence was excited by using the 488-nm band of an argon ion laser scanned through the computer-controlled galvanometer mirrors of a laser scan confocal microscope (Lasersharp MRC-500; Bio-Rad). Emitted fluorescence passed through a long-pass filter (515-nm cut-on) and was collected by a photomultiplier tube. The majority of the images for the $[Ca^{2+}]_{cyt}$ studies were acquired and processed with a CLSM-FLUOVERT system with an inverted microscope (Leica Lasertechnik, Heidelberg). The average number of scans collected per image (time point) was 8 or 10. Changes in fluorescence could only be quantified in guard cells that did not move during the course of the imaging period. As fluorescence did not change in untreated stomata during the experimental period, it was assumed that incomplete dye hydrolysis was not a problem. Photobleaching controls were made by comparing intracellular fluorescence intensities of scanned and adjacent areas at the end of each experiment (14), while dye leakage was assessed by comparing background levels of extracellular fluorescence at the beginning and end of each experiment.

Calculation of Ca^{2+} and pH Levels. Ca^{2+} levels were estimated by ionophore addition [20 μM A23187 in 10 mM Hepes (pH 7–7.4) with 5 or 50 mM KCl] to determine fluorescence limits. The upper fluorescent limit (F_{max}) is determined by adding Ca^{2+} to saturate fluo-3 in the cytosol, while the lower limit (F_{min}) is established by the addition of Mn^{2+}, which displaces Ca^{2+} from the dye and thereby quenches its fluorescence. $[Ca^{2+}]_{cyt}$ was calculated from the equation $[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F)$, where K_d is ≈0.4 μM and fluorescent enhancement upon Ca^{2+} binding is ≈36-fold (14, 20). In a single wavelength mode, the BCECF fluorescence can only be quantitated indirectly *in vivo*. F_{max} and F_{min} were established in the presence of the H^+/K^+ exchanger nigericin in alkaline (20 mM Hepes, pH 8.1) or acid (20 mM Pipes, pH 5.8) buffer containing 50 mM KCl (14, 21). Changes in pH_{cyt} can be calculated as a linear relationship between pH and fluorescence intensity over the pH ranges 6.4–7.6 (21).

RESULTS

Effects of Growth Regulators on Stomatal Aperture. *Paphiopedilum* spp. contain achlorophyllous guard cells (17) and thus lack the red-light response (18), but the guard cells contain the blue-light receptor, and stomata open in response to blue light (18). The effects of growth regulators and other compounds on *P. tonsum* stomata are shown in Table 1. As the changes in stomatal aperture of *Paphiopedilum* spp. are small, stomata with a pore width of >1.5–2 μM were considered as open. Although the stomata did not all respond to treatments as determined by our assay procedures, the effects of these compounds were similar to those seen in other species. Thus, opening occurred in response to IAA, kinetin, FC, and acetic acid, while closure resulted from treatment with ABA or procaine. The effective concentrations of the various compounds were an order of magnitude higher than observed with other species and may be a pecularity of *Paphiopedilum*. The lack of response of some guard cells could reflect either the inherent heterogeneity of stomatal responses (3) or the tardy movement of *P. tonsum* stomata as the epidermal strips were taken from the same area of the leaf and often the pore widths in treated strips were different from the control treatments. The tardy response of the guard cells is advantageous in confocal scanning optical microscopy experiments, as movement is at a minimum during the first minutes after treatment. This reduces the possibility of mistaking artifactual changes in fluorescence (from specimen movement, shifts in focal plane) for changes due to alterations in concentrations of H^+ or Ca^{2+}.

Effects of Growth Regulators on Guard Cell $[Ca^{2+}]_{cyt}$. After epidermal strips had been loaded with fluo-3, the effects of several growth regulators were tested on guard cell $[Ca^{2+}]_{cyt}$. Guard cell $[Ca^{2+}]_{cyt}$ was commonly at a basal level of 0.22 ± 0.015 μM (n = 21) and ranged from 0.05 to 0.3 μM. All compounds tested were added in incubation buffer to avoid the effects of changes in external pH on the guard cells.

A small increase in $[Ca^{2+}]_{cyt}$ was observed in guard cells of open stomata after addition of ABA (Table 2). Increases of 1.5- to 3-fold were observed in 7 of 10 experiments. No change in the fluorescence intensity of fluo-3 was observed in the other three experiments. The increase in $[Ca^{2+}]_{cyt}$ in response to ABA is similar to that reported for guard cells of *Commelina communis* (9). However, another report (11) indicates that prolonged increases in $[Ca^{2+}]_{cyt}$ in response to ABA are atypical and that increases are more likely to be oscillatory. In our experiments, images were taken at discrete intervals, and we may have failed to detect transient in-

Table 1. Effect of treatments on stomatal pores

Agent conc., mM	Open stomata,* %					
	Opening agent†				Closing agent‡	
	IAA	Kinetin	FC	HOAc	ABA	Procaine§
0	23 ± 4	25 ± 2	22 ± 3	25 ± 4	58 ± 4	50 ± 4
0.01	26 ± 8	24 ± 5	30 ± 3	—	48 ± 9	48 ± 3
0.05	—	—	45 ± 3	—	—	—
0.10	40 ± 3	38 ± 8	—	—	40 ± 7	40 ± 2
1.0	49 ± 10	40 ± 7	—	—	35 ± 9	35 ± 4
5.0	—	—	—	52 ± 3	—	—

*Stomata with a pore width > 1.5–2 μm are considered to be open. Data are means ± SEM, and >30 stomata from more than six different strips were assessed 45–60 min after treatment.

†Freshly peeled strips were incubated in incubation buffer containing the appropriate treatment.

‡These strips were first incubated in opening buffer to open stomata before treatment in incubation buffer.

§The incubations for procaine were made in 10 mM Tris 10 mM Mes (2-[*N*-morpholino]ethane sulfonic acid), pH 7.4.

Table 2. Effect of treatments on guard cell $[Ca^{2+}]_{cyt}$

Treatment*		Time, min	$[Ca^{2+}]_{cyt}$,† μM
Agent	Conc., μM		
ABA	80	1	0.24 ± 0.01
		3	0.37 ± 0.03
		7	0.53 ± 0.04
IAA	50	1	0.29 ± 0.05
		7	0.51 ± 0.03
Kinetin	70	1	0.26 ± 0.05
		7	0.48 ± 0.09
FC	25	1	0.27 ± 0.03
		7	0.38 ± 0.03

*Epidermal strips were loaded with fluo-3. Those used in ABA treatments were exposed to blue light and low-CO_2 air in opening buffer to open the stomata prior to loading with fluo-3.
†$[Ca^{2+}]_{cyt}$ is calculated from fluorescence intensity as described. Data are means ± SEM of three or more experiments at each time point. The basal $[Ca^{2+}]_{cyt}$ ranged from 0.1 to 0.25 μM in these experiments.

creases in $[Ca^{2+}]_{cyt}$ in the three experiments where no change was seen. Our results provide further evidence of the variable $[Ca^{2+}]_{cyt}$ response to ABA in guard cells.

Both IAA and FC have been shown to raise $[Ca^{2+}]_{cyt}$ in a variety of cell types (13, 14), but their effect on guard cell $[Ca^{2+}]_{cyt}$ is unknown. As these compounds, along with kinetin, induce stomatal opening, we tested their effect on guard cell $[Ca^{2+}]_{cyt}$. After treatment with IAA or FC, guard cell $[Ca^{2+}]_{cyt}$ increased 1.5- to 2-fold (Table 2). The increase was seen in all cases where IAA was applied and in five of eight experiments where FC was added. No change in fluorescence intensity was observed in the remaining cases. The increase in $[Ca^{2+}]_{cyt}$ in guard cells after addition of IAA or FC is similar to that reported for epidermal and cortical cells of maize coleoptiles (13, 14), maize roots, and parsley hypocotyls (14).

Kinetin also caused an increase in guard cell $[Ca^{2+}]_{cyt}$ (Table 2 and Fig. 1). In the experiment depicted in Fig. 1, $[Ca^{2+}]_{cyt}$ rose from 0.28 μM at 1 min to 0.62 μM at 10 min after application of 50 μM kinetin. Guard cell $[Ca^{2+}]_{cyt}$ rose 1.3- to 2.5-fold within 12 min in 28 of 34 treatments after application of 10–100 μM kinetin. To our knowledge, this is the first report of an increase of $[Ca^{2+}]_{cyt}$ in response to kinetin. Previously it has been shown that the cytokinin, benzyladenine, increased $^{45}Ca^{2+}$ uptake in *Amaranthus* protoplasts (22).

Effect of Growth Regulators on pH_{cyt}. As the opening (IAA, FC, and kinetin) and closing (ABA) agents all raised $[Ca^{2+}]_{cyt}$ to similar levels, it suggests that further factors influence guard cell turgor. One such factor is pH_{cyt}, which could affect the conformation of Ca^{2+}-modulated target proteins. ABA raises pH_{cyt} (14) whereas both IAA and FC induce cytosolic acidification (13–16, 23) in maize coleoptiles. We tested the effects of these compounds on guard cell pH_{cyt}.

The addition of 20–100 μM kinetin to epidermal strips loaded with BCECF resulted in decreased pH_{cyt} of the guard cells by 0.1–0.4 unit within 10 min (Table 3), and most of the change occurred during the first 5 min. Cytosolic acidification was seen in 17 of 19 experiments. An example is shown in Fig. 2, where pH_{cyt} of the guard cells fell ≈0.4 pH unit during the course of the experiment.

Both IAA and FC at 50 μM and 25 μM, respectively (concentrations that consistently opened stomata of *P. tonsum*; see Table 1), reduced pH_{cyt} of guard cells by 0.2–0.4 unit within 5 min of application (Table 3). Their effect on pH_{cyt} of guard cells is similar to that previously reported with microelectrodes (13, 15, 16, 23) or BCECF (14) in maize coleoptiles. However, in all experiments where a decrease in pH_{cyt} of guard cells occurred, a concomitant increase in dye

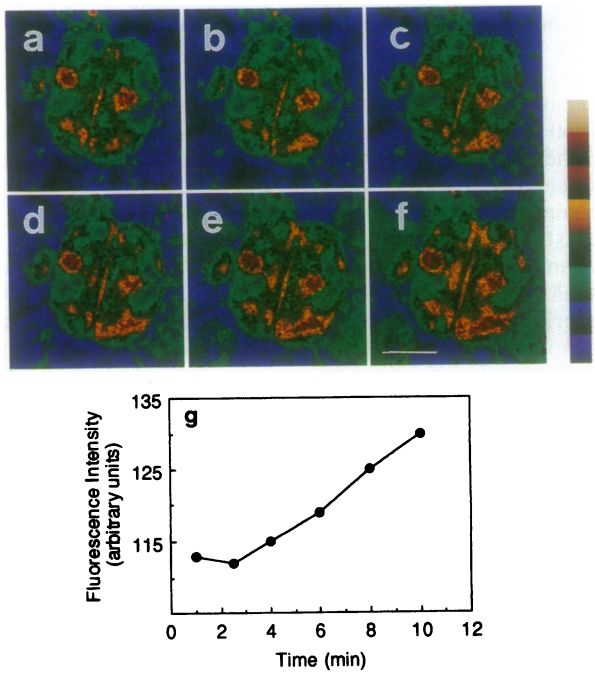

FIG. 1. Changes in $[Ca^{2+}]_{cyt}$ after addition of kinetin. (*a–f*) Fluorescence video images were taken at 1 min (*a*), 2.5 min (*b*), 4 min (*c*), 6 min (*d*), 8 min (*e*), and 10 min (*f*) after the addition of 50 μM kinetin (Sigma) in incubation buffer. The initial image (*a*) was taken with the guard cells focused to their highest intensity, leaving the epidermal cells out of focus. The color bar represents a range of $[Ca^{2+}]$ from 0.01 μM (purple/bottom) to 10 μM (white/top). (Bar = 25 μm.) (*g*) Time plot of fluo-3 fluorescence intensity (arbitrary units) of the stomata in the video images (*a–f*).

fluorescence was evident in the surrounding epidermal cells (as can be seen in Fig. 2). The apparent increase in pH_{cyt} of the epidermal cells would be on the order of 0.05–0.1 pH unit. This is odd, as pH_{cyt} decreased in all other cells exposed to these compounds (Fig. 2; refs. 13–16, 23). It could reflect a different response on part of the mature epidermal cells (taken from fully expanded leaves) as well as a peculiarity of the orchid. Epidermal cells in *Paphiopedilum* spp. have considerably lower levels of K^+ present than other species (24). As high external K^+ is required for stomatal movement (24), the incubation buffer contained 50 mM KCl, which is the source of K^+ to open the guard cells in response to IAA, kinetin, or FC. It is possible that the epidermal cells respond more rapidly to decreased pH_{cyt} and the associated activation of the H^+ pump by enlisting the K^+/H^+ symport, which would result in net K^+ accumulation and alkalinization. Such a mechanism operates in *Neurospora* in response to acid loading (25) and may account for K^+ accumulation in guard cells of *V. faba* stimulated by FC (26, 27).

Table 3. Effect of treatments on guard cell pH_{cyt}

Treatment*		ΔpH_{cyt},† pH unit
Agent	Conc., μM	
Kinetin	70	−0.18 ± 0.04
IAA	50	−0.21 ± 0.05
FC	25	−0.27 ± 0.07
ABA	80	+0.19 ± 0.05
Procaine	1000	+0.33 ± 0.07

*Epidermal strips were loaded with BCECF. Those used in ABA and procaine treatments were exposed to blue light and low-CO_2 air in opening buffer to open the stomata prior to loading with BCECF.
†ΔpH_{cyt} is calculated from the change in fluorescence intensity during the 5- to 7-min experimental period. Data are means ± SEM of three or more experiments.

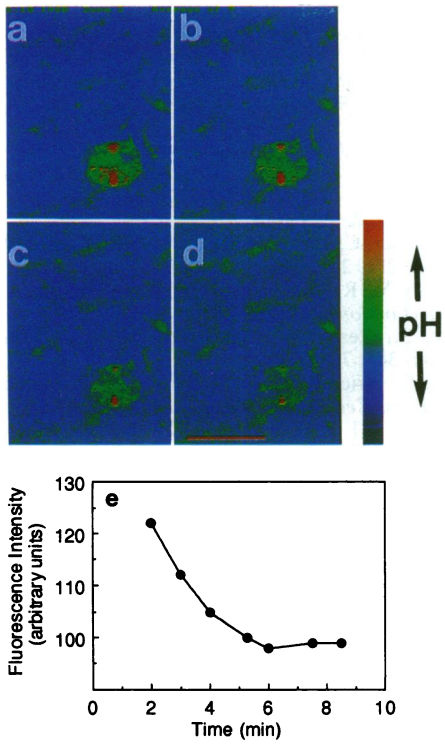

FIG. 2. Changes in pH_{cyt} after addition of kinetin. (*a–d*) Fluorescence video images were taken at 2 min (*a*), 3 min (*b*), 4 min (*c*), and 5.25 min (*d*) after the addition of 100 μM kinetin in incubation buffer. The initial image (*a*) was adjusted so that the highly fluorescent spots of the stomata were at their brightest, leaving the epidermal cells out of focus. The color bar represents a pH range from 6 (pale blue) to 8 (crimson) for the stomata. (Bar = 100 μm.) (*e*) Time plot of BCECF fluorescence intensity (arbitrary units) of the stomata in the video images in *a–d* and for the following few minutes of the experiment when no further change was observed.

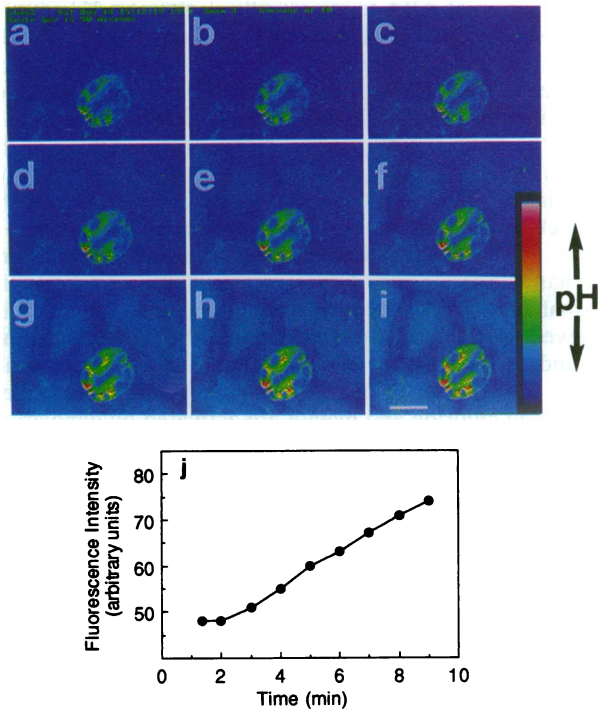

FIG. 3. Changes in pH_{cyt} of an open stomata after the addition of ABA. (*a–i*) Fluorescence video images of two guard cells were taken at 1.33 min (*a*), 2 min (*b*), 3 min (*c*), 4 min (*d*), 5 min (*e*), 6 min (*f*), 7 min (*g*), 8 min (*h*), and 9 min (*i*) after the addition of 100 μM ABA (mixed isomers; Sigma) in incubation buffer. The initial image (*a*) was taken with the guard cells focused to their highest fluorescence intensity, leaving the epidermal cells out of focus. The color bar represents a pH range from 6 (pale blue) to 8 (pink) for the stomata. (Bar = 50 μm.) (*j*) Time plot of BCECF fluorescence intensity (arbitrary units) of the stomata in video images in *a* to *i*.

Fig. 3 shows the change in pH_{cyt} of a single open stomata after treatment with 100 μM ABA. There is a steady increase in guard cell pH_{cyt} up to $\approx$0.3 unit over 8 min. Guard cell alkalinization in response to 10–100 μM ABA was observed within 2 min in 13 of 14 experiments (Table 3). The responses ranged from 0.04 (in 2 cases) to 0.3 unit (in 3 cases) over 5 min, and the majority of the increases were in the range of 0.1–0.2 pH unit. An increase in pH_{cyt} also occurred in the surrounding epidermal cells (increasing background fluorescence as in Fig. 3), which is in accordance with the action of ABA on maize coleoptiles and roots and on parsley hypocotyls (14).

An alkalinizing effect of the weak base procaine has been reported for several types of plant cells (14, 15, 23). Procaine (1 mM) also rapidly alkalinizes guard cells, where increases ranged from 0.1 to 0.4 pH unit in eight stomata observed (Table 3). The effect of procaine on pH_{cyt} was observed within 5 min, and stomatal closure was observed after 1 hr (Table 1). However, no increase in $[Ca^{2+}]_{cyt}$ was observed in guard or epidermal cells in response to procaine (data not shown), and this is consistent with its effect in *Sinapis alba* root hairs where a slight decrease in $[Ca^{2+}]_{cyt}$ was seen (23).

DISCUSSION

Several stimuli known to induce stomatal opening also increase H^+ extrusion from guard cells or protoplasts (28–31). Proton extrusion is often associated with decreased pH_{cyt} (16, 23). We show that kinetin (Fig. 2), IAA, and FC, compounds that enhance stomatal opening and H^+ extrusion, also stimulate cytosolic acidification in guard cells. Weak acids have also been shown to decrease pH_{cyt} (15), and 15–20% of stomata of *P. tonsum* open in response to acetic acid (Table 1). The proportion of open stomata is similar to that seen with treatments of kinetin, IAA, or FC (Table 1). Kinetin (Fig. 1), IAA, and FC all increased $[Ca^{2+}]_{cyt}$ within 7 min in guard cells (Table 2). Weak acids have also been shown to raise $[Ca^{2+}]_{cyt}$ coincidently with cytosolic acidification in maize coleoptiles (13, 23). Therefore, it appears that stomatal opening is preceded by a decrease in pH_{cyt}, an increase in H^+ extrusion, and an increase in $[Ca^{2+}]_{cyt}$ in the guard cells, although the order of these events is still uncertain.

It has been postulated that stomatal closure is triggered by an increase of Ca^{2+} influx at the plasmalemma (1). A nonselective Ca^{2+}-permeable channel is stimulated in guard cell protoplasts in response to ABA, and this is concomitantly accompanied by transient increases in $[Ca^{2+}]_{cyt}$ (12). Similar transient increases in $[Ca^{2+}]_{cyt}$ have been reported in guard cells, but these were not always seen although the stomata closed in response to ABA (11). A more prolonged (several min) transient increase in $[Ca^{2+}]_{cyt}$ is also seen in guard cells (Table 2; ref. 9). Stomatal closure occurs when "caged Ca^{2+}" or "caged inositol trisphosphate" is released within guard cells and $[Ca^{2+}]_{cyt}$ increases to >0.6 μM (10), which suggests that intracellular Ca^{2+} stores are also an important source of calcium. Clearly, an increase in $[Ca^{2+}]_{cyt}$ is an important trigger in stomatal closure. We show that guard cell alkalinization occurs in response to ABA (Fig. 3) and also procaine (a weak base). ABA raised guard cell $[Ca^{2+}]_{cyt}$ in our experiments and this confirms previous work (9, 12); procaine did not, although both ABA and procaine induced stomatal closure (Table 1).

Application of ABA to guard cells also affects K^+ flux (1, 32). Inward-rectifying K^+ channels are inactivated (32, 33), while a leak current and outward-rectifying K^+ channels are activated (32). Release of caged inositol trisphosphate gives rise to increased $[Ca^{2+}]_{cyt}$ in guard cells (10) and also inactivates the inward-rectifying K^+ channels while activating the leak current (33). Although ABA stimulates the outward-rectifying K^+ channels, this is Ca^{2+}-independent (34), and the channels are not affected by either inositol trisphosphate (33) or Ca^{2+} (35). ABA could directly gate the outward-rectifying K^+ channel as suggested (36). However, for this to occur, the plasmalemma must be depolarized. Depolarization could be achieved by Ca^{2+} influx after activation of a Ca^{2+} channel (12) and/or by cytosolic alkalinization (Fig. 3), which would reduce the activity of the proton pump. Anionic channels conducting chloride and malate are activated by depolarization, particularly in the presence of nucleotides and elevated $[Ca^{2+}]_{cyt}$ (37, 38), and this would lead to loss of anions and further depolarization. Thus, increases in both pH_{cyt} and $[Ca^{2+}]_{cyt}$ would have a synergistic effect on the guard cell's capability to depolarize its plasmamembrane. Even if the increase in the pH_{cyt} is not accompanied by an increase in the $[Ca^{2+}]_{cyt}$, there would also be a net depolarization-dependent salt loss from the guard cells (and therefore stomatal closure) although at a reduced rate. This is consistent with our results.

Opening of the stomata by weak acids (Table 1) can also be explained in this context. The weak acids cross the membrane in the uncharged form and dissociate in the cytosol, thereby decreasing the pH. This acidification would stimulate the proton pump, which would hyperpolarize the membrane (15). Hyperpolarization to more negative potentials than the equilibrium potential for K^+ would lead to K^+ accumulation via the inward-rectifying channel if the $[Ca^{2+}]_{cyt}$ is not increasing. However, lowering pH_{cyt} has been associated with an increase in $[Ca^{2+}]_{cyt}$ (13, 23), which would inactivate the inward-rectifying K^+ channels (33, 35). Uptake of K^+ can be against the thermodynamic gradient (26, 27), and this may occur by the K^+/H^+ symport as in *Neurospora* (25). The cytosolic accumulation of the K^+ salt of the weak acid would lead to water accumulation in the guard cells by osmosis and to the opening of the stomata. Both IAA and FC are known to acidify the cytosol (13–16) and cause membrane hyperpolarization in maize cells (13, 15, 16), and FC can stimulate K^+ uptake against the thermodynamic gradient in *V. faba* guard cells (26, 27). The growth regulators kinetin (Fig. 2), IAA, and FC also acidify the guard cell cytosol and open the stomata (Table 1; refs. 5–7). The mechanism of action might involve stimulation of malic acid production in the cytosol (39, 40), which is then responsible for acidification and membrane hyperpolarization. This hyperpolarization in turn would lead to K^+ accumulation by the mechanism described above and to osmotic changes resulting in the opening of the stomata.

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1. MacRobbie, E. A. C. (1988) *Bot. Acta* **101**, 140–148.
2. Darwin, F. (1898) *Phil. Trans. R. Soc. London Ser. B* **190**, 531–621.
3. Mansfield, T. A., Hetherington, A. M. & Atkinson, C. J. (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 55–75.
4. Schulze, E.-D., Turner, N. C., Gollan, T. & Shackel, K. (1987) in *Stomatal Function*, eds. Zeiger, E., Farquhar, G. D. & Cowan, I. R. (Stanford Univ. Press, Stanford, CA), pp. 311–321.
5. Pemadasa, M. A. (1982) *New Phytol.* **90**, 209–219.
6. Turner, N. C. & Graniti, A. (1969) *Nature (London)* **223**, 1070–1071.
7. Incoll, L. D. & Whitelam, G. C. (1977) *Planta* **137**, 243–245.
8. Mittelheuser, C. J. & Van Steveninck, R. F. M. (1969) *Nature (London)* **221**, 281–282.
9. McAinsh, M. R., Brownlee, C. & Hetherington, A. M. (1990) *Nature (London)* **343**, 186–188.
10. Gilroy, S., Read, N. D. & Trewavas, A. J. (1990) *Nature (London)* **346**, 769–771.
11. Gilroy, S., Fricker, M. D., Read, N. D. & Trewavas, A. J. (1991) *Plant Cell* **3**, 333–344.
12. Schroeder, J. I. & Hagiwara, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9305–9309.
13. Felle, H. (1988) *Planta* **174**, 495–499.
14. Gehring, C. A., Irving, H. R. & Parish, R. W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9645–9649.
15. Brummer, B., Bertl, A., Potrykus, I., Felle, H. & Parish, R. W. (1985) *FEBS Lett.* **189**, 109–114.
16. Felle, H., Brummer, B., Bertl, A. & Parish, R. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8992–8995.
17. Nelson, S. D. & Mayo, J. M. (1975) *Can. J. Bot.* **53**, 1–7.
18. Zeiger, E., Assmann, S. M. & Meidner, H. (1983) *Photochem. Photobiol.* **38**, 627–630.
19. Mayo, J. M. & Ehret, D. (1979) *Can. J. Bot.* **58**, 1202–1204.
20. Kao, J. P. Y., Harootunian, A. T. & Tsien, R. Y. (1989) *J. Biol. Chem.* **264**, 8179–8184.
21. Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196.
22. Elliot, D. C. & Yuguang, Y. (1989) *Plant Sci.* **65**, 243–252.
23. Felle, H. (1989) in *Second Messengers in Plant Growth and Development*, eds. Boss, W. F. & Morré, D. J. (Liss, New York), pp. 145–166.
24. Willmer, C. M., Rutter, J. C. & Meidner, H. (1983) *J. Exp. Bot.* **34**, 507–513.
25. Blatt, M. R. & Slayman, C. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2737–2741.
26. Clint, G. M. & Blatt, M. R. (1989) *Planta* **178**, 495–508.
27. Blatt, M. R. & Clint, G. M. (1989) *Planta* **178**, 509–523.
28. Raschke, K. & Humble, G. D. (1973) *Planta* **115**, 47–57.
29. Gepstein, S., Jacobs, M. & Taiz, L. (1982) *Plant Sci. Lett.* **28**, 63–72.
30. Assmann, S. M., Simoncini, L. & Schroeder, J. I. (1985) *Nature (London)* **318**, 285–287.
31. Edwards, M. C., Smith, G. N. & Bowling, D. J. F. (1988) *J. Exp. Bot.* **39**, 1541–1547.
32. Blatt, M. R. (1990) *Planta* **180**, 445–455.
33. Blatt, M. R., Thiel, G. & Trentham, D. R. (1990) *Nature (London)* **346**, 766–769.
34. MacRobbie, E. A. C. (1990) *Proc. R. Soc. London Ser. B* **241**, 214–219.
35. Schroeder, J. I. & Hagiwara, S. (1989) *Nature (London)* **338**, 427–430.
36. Schauf, C. L. & Wilson, K. J. (1987) *Biochem. Biophys. Res. Commun.* **145**, 284–290.
37. Keller, B. U., Hedrich, R. & Raschke, K. (1989) *Nature (London)* **341**, 450–453.
38. Hedrich, R., Busch, H. & Raschke, K. (1990) *EMBO J.* **9**, 3889–3892.
39. Humble, G. D. & Raschke, K. (1971) *Plant Physiol.* **48**, 447–453.
40. Assmann, S. M. & Zeiger, E. (1987) in *Stomatal Function*, eds. Zeiger, E., Farquhar, G. D. & Cowan, I. R. (Stanford Univ. Press, Stanford, CA), pp. 163–193.